U.S. PATENT APPLICATION OF WILLIAM E. MARSHALL

METHODS OF MODULATING THE IMMUNE SYSTEMS OF ANIMALS WITH COMPOSITIONS OF PRODUCTS RELEASED BY STRESSED MICROORGANISMS

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METHODS OF MODULATING THE IMMUNE SYSTEMS OF ANIMALS WITH COMPOSITIONS OF PRODUCTS RELEASED BY STRESSED MICROORGANISMS

This application is a continuation-in-part of co-pending U.S. Patent Application Serial No. 08/376,175, filed on January 20, 1995, which is a continuation-in-part of U.S. Patent Application Serial No. 08/059,745, filed on May 11, 1993, now abandoned.

FIELD OF THE INVENTION

This invention relates to methods of modulating immune responses of animals or humans and to biological compositions for modulating such immune responses. More particularly, the invention relates to methods of modulating immune responses of animals or humans by administering effective amounts of stress response factors (SRFs), or partially purified fractions thereof effective to modulate immune responses.

BACKGROUND OF THE INVENTION

Macrophages and monocytes, sometimes called "sentry cells," are large phagocytic cells of the reticuloendothelial system which engulf and digest or phagocytize cells, microorganisms or other foreign materials in the bloodstream and tissues of humans and animals. As used hereafter "animal" refers to humans and animals, and includes mammals, birds and fish.

Macrophages reside in tissues where infections can originate such as in the oral, pharyngeal and alveolar cavities, the gastrointestinal tract, urethra, vagina, etc. Monocytes are

present in the blood and are immature macrophages. It is believed that exposure to external stimulants, macrophages begin producing cytokine-based signals that report the nature of the stimulant to the associated immune system, as well as certain organ tissue cells.

Thus in their sentry role, macrophages survey the host's internal and external environments, and through the release of approximately 75 different molecules including Interleukin-1 ("IL-1") and Tumor Necrosis Factor ("TNF"), signal the nature of the detected foreign material to other immunocytes.

Although not entirely understood, IL-1 and TNF play a critical role in normal host resistance to infections and the growth of malignant tumors, serving as immunostimulants and as mediators of the inflammatory response. However, over-production has been implicated in a number of pathological conditions including septic shock.

The outer membranes of Gram-negative bacteria contain lipopolysaccharide (LPS or endotoxin), a large molecule consisting of three covalently linked parts: lipid A, polysaccharide core and O-antigens. When a macrophage is exposed to LPS, it releases cytokines including IL-1. When high levels of IL-1 are injected into a host animal, proinflammatory effects are observed followed by acute phase inflammation marked by molecular changes in many tissues. A state of shock, remarkably similar to septic shock, ensues followed by death.

U.S. Patent No. 4,975,467 teaches methods by which synthetic compounds can be used to inhibit the release of IL-1 and thereby

alleviate its mediated pathophysiological conditions. U.S. Patent No. 5,055,447 provides methods and compositions for the treatment or prophylaxis of septic shock by using transforming growth factor-beta. That patent illustrates the complexity of the cytokine signals for thymocyte mitogenesis and teaches the use of a complex mixture of cytokines and other pharmacologically active compounds for control of shock.

U.S. Patents Nos. 5,041,427 and 5,158,939 teach the use of naturally occurring non-toxic LPS from *R. spaeroides*, ATCC 17023, to protect animals from toxic LPS. U.S. Patent No. 5,157,039 supports the clinical need for controlling IL-1 release by macrophages by teaching the use of 2-quinolinyl methoxy compounds to inhibit a family of interleukins. Separately, U.S. Patent No. 5,082,838 teaches the use of sulfur-containing fused pyrimidine derivatives to inhibit IL-1 release to aid in the prevention of inflammation resulting from bactremic infections.

It is theorized that balanced activation of macrophages leads to the synthesis and release of cytokines in a profile that would restore homeostasis to the immune system rather than to initiate a pathophysiologic state.

Cytokine production has been induced by exposing macrophages to mechanically produced cell components of a number of Gram-positive pathogens and non-pathogens as well, (Bhakdi, et al., Infec. & Immun. 59: 4614-4620, 1991 and Gold, et al., Infect. & Immun. 49: 731-741, 1985; Archer, et al., U.K. Biochem. Soc. Trans. 19: 404S, 1991).

U.S. Pat. No. 4,347,240 teaches the use of selected strains of *Lactobacillus caseii*, YIT 9018, for treating and/or preventing tumor growth by injection. Injecting viable or killed bacteria is fraught with medical and legal implications.

Mice fed 2.4×10^9 CFUs per day of a mixture of *Lactobacillus acidophilus* and *caseii* for 8 days demonstrated increased resistance to infection by *Salmonella typhimurium*. The lack of colonization by *Salmonella typhimurium* was attributed to the precolonization of these sites by the *Lactobacilli*.

Perdigon demonstrated that feeding milk fermented with a mixture of *Lactobacillus caseii* and *Lactobacillus acidophilus* for 8 days provided complete protection against *Salmonella typhimurium* infection in mice. However, protection was not afforded by feeding milk fermented by one of the two *Lactobacilli*. The reason for the difference was "not clear." In an earlier publication, she concluded that feeding this regimen resulted in increased association of these bacteria with the intestinal mucosa. However, feeding the centrifugal supernatants of the fermented milk to mice for 5 consecutive days also increased their immune response. She concluded that the "degradation of casein and other milk constituents permits higher absorption and antigenic stimulation. Moreover, this effect is increased by the MDP (muramyl-dipeptide) contained in the cell wall of the lactobacilli strains studied." (Immunology 63:17-23, 1988).

No mention is made in Perdigon's publications that the bacteria need to be subjected to stressors and as such release stress-response factors that activate macrophages. No analysis was made for MDP in the supernatant. In addition, centrifugation at 10,000 x g for 5 min will not yield a supernatant that is bacteria-free.

The synthesis of "heat-shock proteins" is recognized as being induced in bacteria, fungi and mammalian cells in response to an increase in culture temperature of 4°C. In December 1994, Retzlaff demonstrated for the first time that heat shock proteins induced cytokine secretion in cultures of peritoneal mouse macrophages, (Infect. & Immun. 62:5689-5693). The tested heat-shock proteins were synthesized by recombinant genes and were not the result of stressing viable bacteria. The research of Retzlaff would indicate that heat-shock proteins with a molecular size less than 10kD do not exist.

It is therefore an object of this invention to provide an effective method for maintaining the homeostasis of and modulating the immune system of animals by modulating the response of antigen-sampling macrophages to pathogens. It is a further object of this invention to employ these methods for the densensitization of macrophages subsequently exposed to LPS which initiates toxic shock and for the activation of an immune response impaired by stress and disease, such as in HIV-positive individuals. It is a further object to use levels of stress response factors or purified fragments thereof which interact with macrophages at physiological ratios typically found in the

host's environment. In the course of our studies on the interaction of macrophages with commensal bacteria, we have found that stressed bacteria -- a normal, frequently occurring condition -- release factors that normally activate macrophages. These factors are released even from non-viable bacteria so long as the killing procedure does not inactivate the enzymes generating the factors.

DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a chromatogram of SRFs or Sephadex G-25; and Fig. 2 shows ^{3}H-thymidine absorption by human PBL + αCD3 exposed to SRFs.

SUMMARY OF THE INVENTION

The invention comprises methods of making stress response factors (SRFs) for modulating immune responses in animals. "Modulating an immune response in an animal" as used herein includes stimulating an immune response by activating macrophages to release cytokines comprising IL-1 and TNF (for example, to prevent or combat infections); desensitizing macrophages to prevent over-stimulation leading to both local inflammation (for example, rheumatoid arthritis) or systemic inflammation (for example, septic shock); and down-regulating or paralyzing macrophages to prevent their contributory role of cytotoxic macrophages in killing antibody-coated T cells (for example, HIV infections). SRFs administered orally or parenterally at low levels stimulate the immune system by activating macrophages to

release cytokines in particular IL-1 and TNF that are required to initiate an immune response such as in preventing or reducing infections. The administration of higher levels of SRFs counteracts the pathologic role of macrophages in the inflammatory response, locally (for example, rheumatoid arthritis) or systemically (for example, septic shock). Higher levels of SRFs also inhibit the contributory role of cytotoxic macrophages in the killing of antibody-coated T cells (for example, in HIV infections). The methods comprise:

(a) growing a selected bacteria in a media outside of the animal to a selected level of viability;

(b) stressing the selected bacteria thereby initiating the release of SRFs; and

(c) collecting the SRFs.

Preferably, the stressing of the selected bacteria to induce release of SRFs is accomplished by reducing the bioavailability of nutrients to said bacteria. Most preferably, this is accomplished by one or more of the following methods after propagating bacteria to the selected level of viability: (i) altering the pH of the media to affect the bioavailability of nutrients in the media; (ii) removing nutrients from the media; (iii) reducing the volume of the media; (iv) removing the bacteria from the media by centrifugation and suspending the bacteria in a non-nutritive isotonic solution; and (v) adding additional bacteria to said media. Most preferably, the method of stressing the bacteria to release SRFs is by removing the

bacteria from the media and suspending the bacteria in a non-nutritive isotonic solution comprising 0.9% sodium chloride.

The invention further comprises compositions for modulating the immune system of animals comprising an effective amount of SRFs sufficient to activate macrophages in said animals to release cytokines comprising IL-1 and TNF. Preferably, the compositions comprise SRFs produced by stressed bacteria which are commensal to the animals or have co-evolved in association with the animals. Also, preferably the SRFs are produced by bacteria comprising Gram positive, non-pathogenic bacteria. Most preferably the compositions of the invention comprise SRFs produced from stressed bacteria selected from the family *Lactobacillaceae*.

Further, the compositions of the invention preferably comprise SRFs selected by measuring the immunomodulating capacity of said SRFs for enhancing, desensitizing or suppressing an immune response to a particular immunogen in vitro or in vivo. Additionally, the compositions of the invention comprise SRFs having a molecular weight of less than about 50 kDa and/or preferably less than about 10 kDa. Finally, preferably the compositions of the invention comprise SRFs having an arbitrary unit comprising an optical density of a cell-free suspension of said SRFs greater than 0.001 measured at 255 nm.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to methods of modulating immune responses in animals and humans and biological compositions for modulating such immune responses. Of particular significance is the nature of the source of biological compositions and the conditions under which they are formed. Bacteria associated with animals are the source, and stress is the condition which initiates the release of SRFs, potent immunomodulators.

For purposes of this application the term "SRFs" shall mean stress response factors comprising mixtures of oligomers released when bacterial colonies are stressed. SRFs are obtained by subjecting bacteria to biological, chemical or physical stressors which induce the release of SRFs. One example of "stressed" bacteria is bacteria which have been grown to a maximum level of nutritional sustainability of the growth media, wherein the numbers of the bacteria are increased above the sustainable level of the media. However, it is to be understood that other "stressors" such as altering the pH of the media affect the bioavailability of nutrients, removing nutrients, reducing the volume of the media, adding additional bacteria of the same or different species, etc. could also be employed in the methods of the invention. A sudden decrease in the nutritional sustainability of the bacterial habitat is one example of a stressor which we have successfully employed to initiate the release of SRFs.

The oral or parenteral administration of a preparation of SRFs, or of partially purified fractions thereof, to an animal

can be used to desensitize, enhance or suppress an immune response. Their use would be indicated in clinical conditions requiring the modulation of the monocyte-lymphoid cell interaction. Herein, we present two examples: the suppression of LPS-induced shock by desensitizing macrophages and the enhancement of T cell mitogenicity by preventing the destruction of antibody-coated T cells.

The generation of SRFs is not to be confused with the changes in the steady-state level of a number of cellular proteins generally referred to as heat-shock proteins. SRFs are mixtures of oligomers. An abrupt increase in cell density beyond sustainability is sufficient to initiate their release into the media. Changes in temperature of plus or minus about 5 degrees, do not initiate their release.

Maximum release of SRFs can be achieved by resuspending bacteria at high densities in a non-nutritive isotonic solution. Their appearance can be measured by recording the ultraviolet absorbances at 220, 255, and 280 nm. One arbitrary unit of SRF is defined as an optical density of 0.001 at 255 nm. Absorbance at these wavelengths indicate the presence of organic compounds, nucleotides and aromatic amino acid containing peptides.

SRFs prepared from 15 strains representing bacteria that have co-evolved in association with animals, including humans and birds, were found to have common chemical, physical and biological properties as seen by their releases, the occurrence of Fractions I to IV, their molecular size distributions, their

ultraviolet absorbencies, and their abilities to modulate macrophages as indicated.

The release of SRFs in response to stress appears to be well-conserved among bacteria. In addition, the immunocytes of animals that have co-evolved in association with these strains appear to have adapted an immunologic response in reaction to this external but telling stress. This "learned" adaptation appears to prepare the host to mount an appropriate immune response or return it to homeostasis.

Unfractionated SRFs from all bacteria activate macrophages to release interleukins. However, SRFs from different bacteria exhibit different immunomodulating properties in a tissue culture assay measuring the modification of the macrophage-dependent responses of T lymphocytes to a mitogen, the CD3 specific monoclonal antibody. Partially purified individual fractions administered to mice or assayed in tissue culture assay also demonstrate different immunomodulating properties, as well. Furthermore, the parenteral administration of Fraction I to mice induces fever whereas, a combination of Fractions II, III, and IV does not. Therefore, Fractions II, III and IV alone or in combination can be injected into animals to modulate their immune system without toxic side effects.

Figure 1 shows a typical chromatogram of SRFs on Sephadex G-25. Centrifugation through molecular exclusion filters indicates that SRFs consist of three molecular size classes: 30-50 kDa, (Fraction I), 3-10 kDa, Fraction II), and less than 3 kDa, (Fractions III and IV). The separation of various fractions

of SRFs can readily be achieved by centrifugation of the biomass and passing the supernatant through ultrafiltration membranes with cut offs at the appropriate kDa's. Different bacteria yield components in all of these classes but in different proportions. SRFs released over 12-hour periods from 12 to 120 hrs. yield similar patterns but again, with varying proportions.

Knowing the molecular size range and evaluating the ratios of ultraviolet absorbances suggests that Fraction I is rich in polymers of nucleotides, peptides and peptidoglycans; Fraction II, oligopeptidoglycans and saturated compounds, Fractions III and IV, oligonucleotides and oligopeptides, respectively.

Example 1

Efficient Generation of SRFs

Bacteria were grown in recommended media (Difco, Detroit, MI). *Listeria monocytogenes* was propagated in Brain-Heart-Infusion, (Rosenow, J. Dent. Res. 1:205, 1919) or deMan-Rogosa-Sharpe media, J. Appl. Bact. 23:130, 1960). All bacteria were grown to $2x10^9$ CFUs, chilled and centrifuged at 8000xg, washed twice with cold isotonic saline equal to the volume of the media, centrifuged and resuspended in isotonic saline at $4x10^{10}$ CFUs and incubated at 37°C for 48 hrs. After centrifugation, the supernatant was passed through a sterile 0.22μ filter and an absorbance at 255 nm of 24,000 was recorded. This corresponds to 24,000 arbitrary units per ml. The solution was held refrigerated or frozen.

The quantities of SRFs released by strains of three representative genera illustrate this phenomenon: a Gram-positive commensal, *Lactobacillus caseii*, released 7,000 units/48 hrs.; a Gram-negative, *Escherichia coli*, released 4,000 units/48 hrs.; and an opportunistic Gram-negative pathogen, *Pediococcus aeruginosa* released 4000 units/48 hrs. In addition, the amounts of SRFs released by the following bacteria have been observed to fall within the range of 3000 to 24,000 units over a 48 hr. period: *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus acidophilus*, *Escherichia coli*, *Bifidobacteria coryneform*, *Lactobacillus inocula*, *Salmonella typhimurium*, *Pediococcus acidolactici*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Enterococcus faecium*.

Example 2

Activation of Macrophages by SRFs

The SRFs were prepared from *Lactobacillus caseii*, 393, *Lactobacillus plantarum*, *Pediococcus acidolactici*, *Lactobacillus acidophilus* 4356, *Bifidobacteria coryneform*, and *Enterococcus faecium* according to Example 1 at 2 x 10^9 CFUs for 48 hrs.

A macrophage cell line MonoMac6 originally obtained from Dr. H. W. L. Zeigler-Heitbrock was maintained in RPMI 1640 buffer (Gibco, Grand Island, NY) containing 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2. (Zeigler-Heitbrock, et al., Int. J. Cancer 41:456-461, 1988.)

Mononuclear leucocytes were separated from heparinized peripheral blood by gradient density centrifugation of Ficoll-Hypaque (Sigma Chemicals, St. Louis, MO) and placed in RPMI 1640 supplemented with 5% fetal calf serum in a 24-well plate and incubated for 4 hours to allow attachment of the adherent cells. The macrophages were maintained in RPMI 1640 with 2 mM of L-glutamine.

Aliquots of 0.16 ml of the bacteria-free filtrate containing SRFs were added to 1.44 ml of the macrophage suspension (1x10^6 cells/ml) in complete RPMI and incubated for 2 hrs. at 37°C in an atmosphere of 5% CO_2. The suspension was centrifuged at 500 x g for 5 minutes, the macrophage pellet gently washed with an equal volume of RPMI and resuspended in 1.60 ml of RPMI containing 10% fetal calf serum.

The interleukins, IL-1α and the TNFα were determined in duplicate on 0.2 ml aliquots of the MØ that had incubated for 24 hrs. after having been exposed to the bacterial-free filtrate for 2 hrs. The test procedure and standards supplied by the manufacturer (R & D Systems, Minneapolis, MN) were followed and the amounts of interleukin determined from a standard curve.

Table 1

Bacterial Source of SRFs	IL-1α (p/ml)	TNFα (pg/ml)
Lactobacillus acidophilus	6	42
Lactobacillus caseii	6	56
Lactobacillus plantarum	4	31
Fr II - IV	6	37
Pediococcus acidolactici	7	67
Enterococcus faecium	4	41
Bifidobacteria coryneform	5	85
Staphylococcus aureus	26	62
Pediococcus aeruginosa	17	94
Listeria monocytogenes	13	110
Saline Control	0	2
RPMI Control	0	0

Two examples of useful applications for SRFs are provided herein: an in vivo prevention of septic shock and a tissue

culture demonstration of immune-enhancing and immune-suppressing capacities.

Example 3

Protection of Mice Against Lethal Shock by SRFs

Table 2 shows the level of protection achieved by mice consuming drinking water containing SRFs over a 72-hour period prior to a lethal intraperitoneal injection of LPS derived from *Escherichia coli* O127:B8, (Beutler, et al., Science 229:869, 1985). SRFs from *Listeria monocytogenes, Escherichia coli, Lactobacillus plantarum, Lactobacillus caseii and Lactobacillus acidophilus* were assayed.

Table 2

Units of SRFs consumed daily through drinking water	Percentage of mice surviving after receiving 400 μg of LPS	
600 Units	100%	4/4
1000	75%	3/4
none	0%	0/10

Table 3 shows that protection can also be obtained by the parenteral administration of SRFs 24-48 hours before challenge.

Table 3

Units of SRFs injected interperitoneally in one treatment	Percentage of mice surviving after receiving 400 μg of LPS	
4800 Units	100%	2/2
1200	85%	6/7
300	0%	0/2
none	0%	0/10

The desensitization to LPS by orally administering SRFs is not accompanied by any apparent side effects. Although the parenteral administration is accompanied by slightly ruffled fur and the temporary cessation of eating, these effects can be avoided by injecting combinations of only Fractions II, III, and IV.

Example 4

T cell Mitogenicity Modulation by SRFs

Tissue culture experiments show that SRFs modify the macrophage-dependent responses of an antibody-coated T cell to a mitogen, the CD3 specific monoclonal antibody. This antibody mimics a natural process in which macrophages and T cells form cellular conjugates which enable T cells to mount an immune response. (Leo, et al., J. Proc. Natl. Acad. Sci. 84: 1374, 1987).

Fig. 2 demonstrates that SRFs from *Listeria monocytogenes* 8830 enhance the mitogen response and, therefore would increase

the immune response. On the other hand, SRFs from *Lactobacillus caseii* 393 suppress this response and, therefore, diminish an immune response. The ability of one preparation to compensate for the other as seen in their mixtures indicates that their modes of action share similar pathways. Fraction III of the SRFs from *Listeria monocytogenes* 8830 and Fraction II of the SRFs from *Lactobacillus caseii* 393 are responsible for these opposite activities.

Clinical circumstances exist in which up- or down-regulation of stimulated T lymphocytes is warranted, e.g. in immune suppression and autoimmune diseases, respectively.